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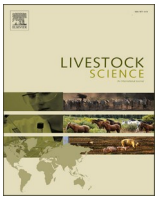

Application of an optimized and validated LC–MS/MS method for the quantification of free 3-nitrotyrosine in plasma, urine and liver tissue of lactating dairy cows

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HIGHLIGHTS

- The transition period is the most critical in the productive cycle of dairy cows
- Increased oxidative stress in transition cows could impair metabolic adaptation
- The biomarker 3-nitrotyrosine (3-NT) is a candidate to evaluate oxidative stress
- We developed a LC-MS/MS method to determine 3-NT in plasma, urine and liver tissue

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ABSTRACT

In dairy cows, the high energy demand and oxygen requirements during the transition period could increase oxidative stress (OS), impairing the challenging metabolic adaptation needed during this period. Also, OS and health could be affected by some environmental conditions such as heat stress. In this sense, 3-nytrotyrosine (3-NT) has been described as a biomarker of OS. Therefore, the aim of this study was to develop and validate a fast, reliable method for the accurate determination of free 3-NT in blood, urine and liver samples by liquid chromatography–tandem mass spectrometry (LC-MS/MS). To this end, during the transition period, 3-NT concentrations were analyzed in samples from dairy cows grouped according to different environmental conditions: cows that gave birth during winter (winter group, WG; n = 18) and cows that gave birth during spring (spring group, SG; n = 18). Additionally, other plasma parameters such as β-hydroxybutyrate acid (BHBA) and glucose concentrations and liver triacylglycerol (TAG) content and aspartate transaminase activity were exhaustively studied. Samples were collected at 21 (±3) days before the expected calving date, and at 7 (±3) and 21 (±3) days after calving. The analysis of the samples involved a purification step by solid-phase extraction. The method developed was validated in terms of linearity, specificity, precision, accuracy, recovery, matrix effect, dilution and analyte stability. The lower limit of quantification of the method was 1.02 ng/mL for plasma, 0.24 mg/L for urine and 0.05 mg/kg for liver tissue. Coefficients of variation for the concentrations tested were < 15% and accuracy was within 85–115%. 3-NT concentration was higher in cows of the SG than in those of the WG in the three matrices evaluated. BHBA and glucose concentrations were higher and lower, respectively, in cows of the SG. BHBA concentration was particularly higher in cows of the SG on day 21 postpartum. Liver TAG content and aspartate transaminase activity were also higher in cows of the SG on the postpartum days. These results suggest that the methodology developed and validated by LC-MS/MS for the quantification of 3-NT in different fluids and liver tissue could be a very useful parameter to evaluate OS in dairy cows during the transition period.

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1. Introduction

During the transition period, defined as the period from three weeks before to three weeks after calving, dairy cows experience metabolic and hormonal changes. These changes involve a negative energy balance, with an increase in lipolysis of adipose tissue and consequently an increase in the systemic concentration of non-esterified fatty acids (NEFAs), which have to be metabolized by the liver (McArt et al., 2013). However, when the NEFA influx exceeds the liver's processing capacity, NEFAs are accumulated and hepatic steatosis or fatty liver may consequently develop (Ospina et al., 2010; Angeli et al., 2019a).

To assess liver function, several studies have measured different metabolites and developed various indices, but have not reached conclusive results (Trevisi et al., 2011; Bertoni and Trevisi, 2013; Zhou et al., 2017). In particular, these studies have focused on acute phase proteins produced by the liver and related to inflammatory states (Bertoni and Trevisi, 2013) because inflammation is related to the production of reactive oxygen species (ROS), which are generated from oxygen metabolism, and whose excessive amounts can be detrimental to cells (Castillo et al., 2001; Celi and Gabai, 2015). To attenuate the effects of ROS and help maintain homeostasis, organisms are equipped with endogenous antioxidant defense systems. However, an imbalance between a high level of ROS and lower availability of antioxidant defenses leads to oxidative stress (OS) (Rios et al., 2017). OS can also be increased by reactive nitrogen species (RNS), which, together with ROS, are known as reactive oxygen and nitrogen species (RONS).

During the transition period of dairy cows, the high energy demand and greater oxygen requirements lead ROS to be generated faster than the neutralization mechanisms of the antioxidant system. Additionally, this situation could be influenced by seasonal patterns. In particular, during summer months, heat stress could generate endocrinological changes, increasing the OS and metabolic challenge during the transition period (Bernabucci et al., 2002; Zachut et al., 2017; Huber et al., 2020).

The study of OS in ruminants is a relatively young field of research. Several studies have addressed the analysis of elements of the antioxidant system of cows, such as superoxide dismutase, catalase and glutathione peroxidase (Gong and Xiao, 2018; Sayiner et al., 2021). In addition, other researches have focused on the pro-oxidant elements. In this sense, and given the high instability and short half-life of RONS, different tools have been developed to assess oxidative damage. In this regard, an alternative is to quantify other biomarkers of OS, which can be classified as molecules that are modified by interactions with ROS in the microenvironment (Ho et al., 2013). One of these modifications is the nitration of tyrosine by peroxynitrites and other RONS, which leads to the formation of the stable end product 3-nitrotyrosine (3-NT) (Li et al., 2015). Since this biomarker is chosen as an elite indicator to measure oxidative stress in other species, it may also be useful to indicate the susceptibility of dairy cattle to several diseases and metabolic disorders during the transition period and under different environmental conditions. Only a few other biomarkers have been analyzed in healthy cows, such as malondialdehyde (MDA) (Castillo et al., 2006) and 8-iso-prostaglandin F2α (Putman et al., 2018).

Free 3-NT has been detected in several biological matrices and fluids, including plasma, serum, urine, cerebrospinal fluid, synovial fluid, tissues, and other biological samples (Radabaugh et al., 2008). Free 3-NT can be detected and quantified by different analytical techniques, including immunochemistry, ultraviolet-visible (UV/Vis) photometry, gas chromatography, and high-performance liquid chromatography (HPLC) coupled to different detectors. All these techniques present advantages and disadvantages, although evidence shows that chromatographic methods associated with mass spectrometry are the most frequently chosen because of their high selectivity and sensitivity, simple sample pretreatment steps, and reliable quantification and confirmation at low concentration levels (Yang et al., 2010; Teixeira et al., 2016, 2017).

The concentration of free 3-NT has been studied most extensively in human plasma. In dairy cows, only a few reports have determined 3-NT plasma concentrations, mainly by enzyme-linked immunosorbent assay (ELISA) (Cigliano et al., 2014). Thus, it would be important to analyze this metabolite in bovine plasma by other techniques such as spectrometry, as well as in other matrixes such as urine or liver tissue, especially in the latter considering the relevance of this organ in the transition of dairy cows. Therefore, the aim of this study was to develop and validate a fast, reliable method by using liquid chromatography with tandem mass spectrometry (LC-MS/MS) for the accurate determination of free 3-NT in plasma, urine and liver tissue from dairy cows. Additionally, 3-NT and other plasma parameters, such as NEFAs, albumin, total bilirubin, cholesterol, β-hydroxybutyrate acid (BHBA) and glucose concentrations, and liver triacylglycerol (TAG) content and aspartate transaminase (AST) activity were evaluated during the transition period and under different environmental conditions, which could increase oxidative metabolic processes and thus affect the animal susceptibility to new infections.

2. Materials and methods

2.1. Animals

The present study was carried out according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching (Federation of Animal Science Societies, 2010) and approved by the Ethics Committee of the Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, protocol number 292/16, Santa Fe, Argentina.

The experiment was carried out in a commercial grazing dairy farm in the locality of Hipatia (31°07′S, 61°03′W), Santa Fe, Argentina. Thirty-six Holstein cows, close to entering their second to fourth lactation, were selected. Eighteen cows gave birth during winter, between June and August, and were grouped in the winter group (WG), whereas other eighteen cows gave birth during spring, between October and

Table 1
Ingredients and chemical composition of the diets given to prepartum and postpartum cows of the experimental herds.

	Prepartum	Postpartum	
		WG	SG
Ingredient (% DM)			
Corn silage	19.52	20.22	20.09
Cracked corn grain	7.18	29.55	18.40
Soybean silage		9.24	6.12
Soybean meal	7.18	5.74	6.56
Wheat grain		6.25	6.22
Wheat silage			3.82
Alfalfa grazing		16.37	36.83
Rye grass grazing		21.67	
Sunflower meal	21.50		
Wheat straw	43.01		
Mineral and vitamin pack[1]	1.60	0.96	0.96
DMI (kg/d)	12.6	20.8	20.9
Chemical			
CP (% DM)	13.8	17.0	16.9
NDF (% DM)	50.8	31.8	29.5
ADF (% DM)	39.4	19.7	19.2
NFC (% DM)	29.4	42.3	42.3
NEl (Mcal/kg)	1.40	1.63	1.63

WG: winter group. SG: spring group. %DM: dry matter basis; ADF: acid detergent fiber; NFC: non-fiber carbohydrate; DMI: dry matter intake.

[1] The prepartum pack contained Ca 11%, P 1.5%, Mg 13%, S 5.5%, Cl 28.5%, Fe 825 ppm, Zn 2183 ppm, Mn 1400 ppm, Cu 335 ppm, I 20 ppm, Se 10 ppm, Co 5 ppm, Vit A 100000 IU/kg, Vit D 27000 IU/kg and Vit E 3350 IU/kg, whereas the postpartum pack contained Ca 22%, Mg 7%, Na 10%, Cl 15%, monensin 1200 ppm, Fe 170 ppm, Zn 4500 ppm, Mn 4700 ppm, Cu 950 ppm, I 75 ppm, Se 10 ppm, Co 10 ppm, Vit A 140000 IU/kg, Vit D 70000 IU/kg and Vit E 1400 IU/kg.

December, and were grouped in the spring group (SG). Diet composition is summarized in Table 1. Before and during the experiments, the animals were clinically checked and showed no evidence of disease such as retained placenta, clinical milk fever, mastitis, metritis, clinical ketosis, displaced abomasum, lameness and/or clinical gastrointestinal disorders.

2.2. Milk yield and body condition score (BCS)

Cows were milked twice a day after parturition and milk production was recorded monthly up to 120 days in milk with milk meters (Waikato Milking Systems, Hamilton, New Zealand). The BCS of animals was recorded based on a 1 to 5 scale with 0.25 intervals, as described by Edmonson et al. (1989).

2.3. Meteorological data

Air temperature and relative humidity data were obtained from a meteorological station located at Instituto Nacional de Tecnología Agropecuaria (INTA, EEA Rafaela, Santa Fe, Argentina) and recorded every 10 minutes using the average of the day. The temperature-humidity index (THI) was determined according to the following equation (NRC, 1971): $THI = (1.8\,x\,T + 32) - (0.55 - 0.0055\,x\,RH)\,x\,(1.8\,x\,T - 26)$, where T corresponds to the air temperature in degrees Celsius and RH corresponds to the relative humidity in percent.

2.4. Sample collection

Samples (blood, urine and liver tissue) were collected at the beginning and at the end of the transition period, and after birth, i.e., 21 (±3) days before the expected calving date, and 7 (±3) and 21 (±3) days after calving. Blood was collected from the jugular vein in tubes with EDTA and cooled at 5 °C. Tubes were centrifuged for 10 min at 2000 g to separate the plasma. Urine samples were collected in sterile bottles by stimulating the area below the vulva. Finally, liver tissue was sampled via puncture biopsy, as previously described (Angeli et al., 2019b) and stored in an antioxidant buffer that consisted of 100 mmol/L diethylenetriaminepentaacetic acid, 50 mmol/L butylated hydroxy toluene in 1% (v/v) ethanol, and 10 mmol/L 3-amino-1,2,4-triazole in 50 mmol/L sodium phosphate buffer, pH 7.4 (Vivekanandan-Giri et al., 2011). All samples were stored at −80 °C until analysis.

2.5. Plasma metabolites and liver TAG measurements

BHBA concentration was determined in whole blood by using reactive strips and a handheld device (Precision Xtra meter, Abbott Diabetes Care Inc., Alameda, CA, USA). Plasma concentrations of NEFAs, glucose, albumin, total bilirubin, cholesterol, AST and gamma-glutamyl transpeptidase (GGT) were determined enzymatically with commercial kits by using an ultra-fast UV/Vis spectrometer SPECTROstar Nano (BMG LABTECH GmbH, Ortenberg, Germany). Plasma NEFA concentrations were determined using the commercial colorimetric kit number FA115, from RANDOX Laboratories LTD (UK), whereas the remaining metabolites and enzymes were determined enzymatically using commercial kits from WIENER Lab (Rosario, Argentina) (Angeli et al., 2019a). Liver TAG content was measured according to Angeli et al. (2019b).

2.6. LC-MS/MS

2.6.1. Chemicals

HPLC grade methanol, acetonitrile (ACN), and water were obtained from J.T. Baker (USA). Formic acid (90 %) was obtained from Cicarelli (Argentina) and Trifluoroacetic acid (TFA) from Biopack (Argentina). For the analytical determination of the compound under study, 3-chloro-L-tyrosine (3-ClT) was used as internal standard, to normalize the losses of the compound of interest during the treatment of samples. 3-NT and 3-ClT were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.6.2. Equipment

A Shimadzu ultra-fast liquid chromatograph (UFLC-XR) (Shimadzu Co., Kyoto, Japan) equipped with a Shimadzu Shim-pack GIST C18 column (3.0 μm, 100 mm x 4.0 mm) and a Shimadzu-pack GIST (G) C18 guard column (3.0 μm, 4.0 × 10 mm) was used. The column temperature was kept at 35 °C and the autosampler temperature at 4 °C. Detection and quantitation were achieved by using a QTrap 3200 triple quadrupole mass spectrometer (AB Sciex) equipped with a turbo electrospray ion source. Data were acquired and processed using ANALYST (version 1.6.1, Applied Biosystems, USA).

2.6.3. Chromatographic and mass spectrometric conditions

Mobile phase A was prepared using 0.1 % formic acid in water, whereas mobile phase B was prepared using 0.1 % formic acid in ACN. The triple-quadrupole mass spectrometer was operated in positive ionization mode, and detection and quantification were performed using multiple reaction monitoring. The curtain gas was set at 20 psi; the nebulizer gas and turbo gas were set at 60 psi and 50 psi respectively; the electrospray voltage was set at 5500 V; and the turbo ion spray source temperature was set at 250 °C. The selected *m/z* transitions were 227.2 → 181.0 and 216.2 → 170.2 for 3-NT and 3-ClT, respectively.

2.6.3.1. Plasma and liver tissue samples. For plasma and liver tissue samples, the flow rate was 0.15 mL/min and the injection volume was 20 μL. The elution profile was as follows: 0–1 min, 2% B; 1–6 min, 2–70% B; 6–6.5 min, 50% B; 6.5–6.6 min, 50–2% B; and 6.6–15 min, 2% B.

2.6.3.2. Urine samples. For urine samples, the flow rate was 0.25 mL/min and the injection volume was 5 μL. The elution was under isocratic conditions (A:B, 60:40, V:V).

2.6.4. Preparation of calibration standards

Stock solutions of 3-NT and the internal standard 3-ClT were prepared in ACN:water (50:50, v/v) at concentrations of 1000 mg/L. Working solutions of 3-NT and 3-ClT were prepared with concentrations of 10 mg/L and 1 mg/L, respectively, by dilution of the corresponding stock solutions with ACN:water (50:50, v/v). All stock solutions were stored at −20 °C until needed and working solutions were freshly prepared daily. Calibration standards were prepared by serial dilution of working solutions from 3-NT in the same solvent and subsequently treated in the same way in each matrix. Quality control samples were prepared with diluted, neat or spiked matrix for the low, medium and high level, respectively, and the limit of quantitation (LOQ) was prepared in a surrogate matrix enriched with the analyte at the proposed lower limit of the calibration range (Houghton et al., 2009).

2.6.5. Sample preparation

2.6.5.1. Plasma. A volume of 1000 μL plasma was spiked with 50 μL of 0.4 mg/L 3-ClT working solution and 1000 μL of 1% formic acid. Samples were mixed before the solid-phase extraction (SPE) procedure. A Sep-Pak Vac C18 cartridge (3 CC, 200 mg) was used for the SPE procedure. The cartridge was conditioned with 2 mL of methanol, 2 mL of methanol with 1 % formic acid, and 2 mL of water with 1 % formic acid. Plasma samples were loaded and washed with 1 mL 1 % formic acid in water, and then with 1 mL water. After drying the cartridge, 3-NT and 3-ClT were eluted with 1 mL of a mixture of ACN:water:formic acid (90:9.9:0.1). The eluted solution was evaporated under a stream of nitrogen, and the residue was re-suspended in 100 μL of water and loaded into a vial with insert for LC-MS/MS analysis.

2.6.5.2. Urine. Urine samples (1000 μL) were spiked with 50 μL of 100

mg/L 3-ClT working solution and 1000 μL of 1 % formic acid. A Sep-Pak Vac C18 cartridge (3 CC, 500 mg) was used for the SPE procedure. The cartridge was conditioned with 2 mL of ACN, 1 mL of a mixture of ACN: water:formic acid (90:9.9:0.1) and 1 mL of water with 0.1 % formic acid. Samples were loaded and washed with 1 mL of water. After drying the cartridge, 3-NT and 3-ClT were eluted with ACN:water:formic acid (90:9.9:0.1, 2 mL) and loaded into a vial with insert for LC-MS/MS analysis. The urinary concentration of 3-NT was then normalized to creatinine by division by the creatinine concentration. Creatinine concentration was determined by using an ultra-fast UV/Vis spectrometer SPECTROstar Nano (BMG LABTECH GmbH, Ortenberg, Germany) and a commercial colorimetric kit from WIENER Lab (Rosario, Argentina).

2.6.5.3. Liver tissue. Liver tissue (100 mg) was homogenized with 50 μL of 1 mg/L 3-ClT working solution. Then, 1 mL of TFA 10 % was added and the mixture was homogenized again for 1 min, prior to the centrifugation at 10000 rpm for 10 min. The aqueous layer was placed into another tube. A volume of 1 mL of TFA 10 % was added to the residue, followed by homogenization for 1 min and centrifugation at 10000 rpm for 10 min, and this aqueous phase was combined with the first aqueous phase obtained to perform the SPE procedure, as described above for urine. The eluate (500 μL) was evaporated to dryness and then reconstituted in water (100 μL) before injection into the UFLC system. The liver concentration of 3-NT was expressed per kg of wet tissue.

2.6.6. Validation procedure

The analytical method proposed for each matrix was validated using a surrogate calibration matrix as proposed by Houghton et al. (2009), due to the fact that the compound under study is found endogenously, and following the validation guide of the Food and Drug Administration (2018).

2.6.6.1. Linearity and LOQ. The calibration curve was prepared by fortifying the surrogate matrix (water). The calibration curve was constructed by plotting the peak area ratio of 3-NT and 3-ClT versus 3-NT concentration. The LOQ was defined as the lowest concentration in the calibration curve that can be measured within acceptable accuracy and precision values.

2.6.6.2. Specificity. The specificity assessment included an analysis of the blank substitute matrix to assess any interference with the analyte and an analysis of the blank matrix to look for interference with the internal standard 3-ClT. The assessment was carried out by comparing the chromatograms with those of the blank and surrogate matrices spiked at the LOQ to detect the presence of interfering peaks.

2.6.6.3. Accuracy and precision. Accuracy (%) and precision (CV %) of the low-, medium-, and high-quality control samples were determined in triplicate and quintuple, respectively.

2.6.6.4. Recovery and matrix effect. The analyte recovery percentage was tested at the LOQ, and low, medium, and high quality control levels in triplicate. It was determined by comparing the ratio of 3-NT and 3-ClT areas in plasma samples enriched with the analyte and subsequently processed with the ratio in plasma samples that were first processed and then enriched. The response of the fortified analyte after treatment provides a relative response value of 100 %, and the response data for the extracted samples containing the analyte show no loss in the extraction process.

The matrix effect (ME) was studied by performing a calibration curve in plasma and another one in the water substitute matrix and comparing the slopes, using the following equation (Moreno-González et al., 2018):

$$\%\ \mathrm{ME} = \left(\frac{\text{calibration curve slope in matrix}}{\text{calibration curve slope in water}} - 1\right) \times 100$$

2.6.6.5. Dilution. Sample dilution is used to bring over-range sample concentrations into the calibration range of the assay. The quality control samples were diluted by over spiking endogenous concentrations of the analyte in the matrix with additional analyte to give an appropriate concentration above the calibration range, in quintuple, and then diluted into the calibration range with the surrogate matrix before sample extraction and analysis. The accuracy and precision of these diluted quality control samples should be demonstrated. Dilutions used during the validation should mimic the expected dilutions in the study.

2.6.6.6. Stability. The short- and long-term stabilities of the analyte in the medium- and high-quality control samples, which were stored at -80 °C, were evaluated in duplicate. Short-term stability, determined as freeze-thaw stability (i.e. stability after three freeze and thaw cycles of at least 12 h of the enriched quality control samples), was studied at 30 days. Long-term stability was studied at six months.

2.6.7. Biological samples

After the optimization and validation procedures, plasma, urine and bovine liver samples were analyzed.

2.7. Statistical analysis

The validation parameters were acquired and processed using ANALYST (version 1.6.1, Applied Biosystems, USA).

The biological variability of 3-NT in dairy cows was statistically analyzed through the statistical software package SPSS 25.0 for WINDOWS (SPSS Inc., Chicago, IL, USA). The distribution of data was tested for normality by using the Kolmogorov-Smirnov test. A repeated-measures analysis was performed using the generalized linear model (GLM) approach with log link function for non-normal variables. For outcome variables with normal distribution, a GLM with linear link function was used. The model consisted of season, time, and season × time as fixed effects, and cow as the random effect. When the S × T interaction was significant, differences between treatments at each time point were tested for significance with a GLM. A value of $P < 0.05$ was considered significant. The results are expressed as mean ± SEM.

Agreement between the 3-NT concentration in the different matrices was expressed as correlation coefficient (r), with the Spearman nonparametric correlation coefficient, in which an r value = 0.68 to 1.00 was rated as a strong or high correlation, an r value = 0.36 to 0.67 as a moderate correlation, and an r value = < 0.36 as a weak correlation.

The sample size was calculated a priori using the software G*Power (version 3.1.9.4; Faul et al., 2007) as follows: repeated-measures within-between interaction analysis (significance level of 0.05, power of 0.8, effect size of 0.4, correlation among repeated measures of 0.5, and non-sphericity correction of 0.5). Based on the sample size test, twenty animals were required.

3. Results

3.1. Method validation

Although 3-ClT is an oxidation product of ROS in human samples, no signal was detected in the bovine blank matrix, so it was here used as an internal standard in bovine samples. In addition, a blank was performed for each biological bovine sample, confirming that there was no 3-ClT signal.

The analytical method was specific for each matrix, differentiating 3-NT and 3-ClT (Figure 1). The matrix effect for plasma and urine was negligible, whereas that for liver was medium, indicating that the slopes of the curve are similar and can be used to quantify the surrogate matrix. The calibration curve of 3-NT was fitted with a $1/x^2$ weighted linear regression over a concentration range of 1.02–11.10 ng/mL for plasma, 0.24–19.31 mg/L for urine and 0.05–2.04 mg/kg for liver tissue. The

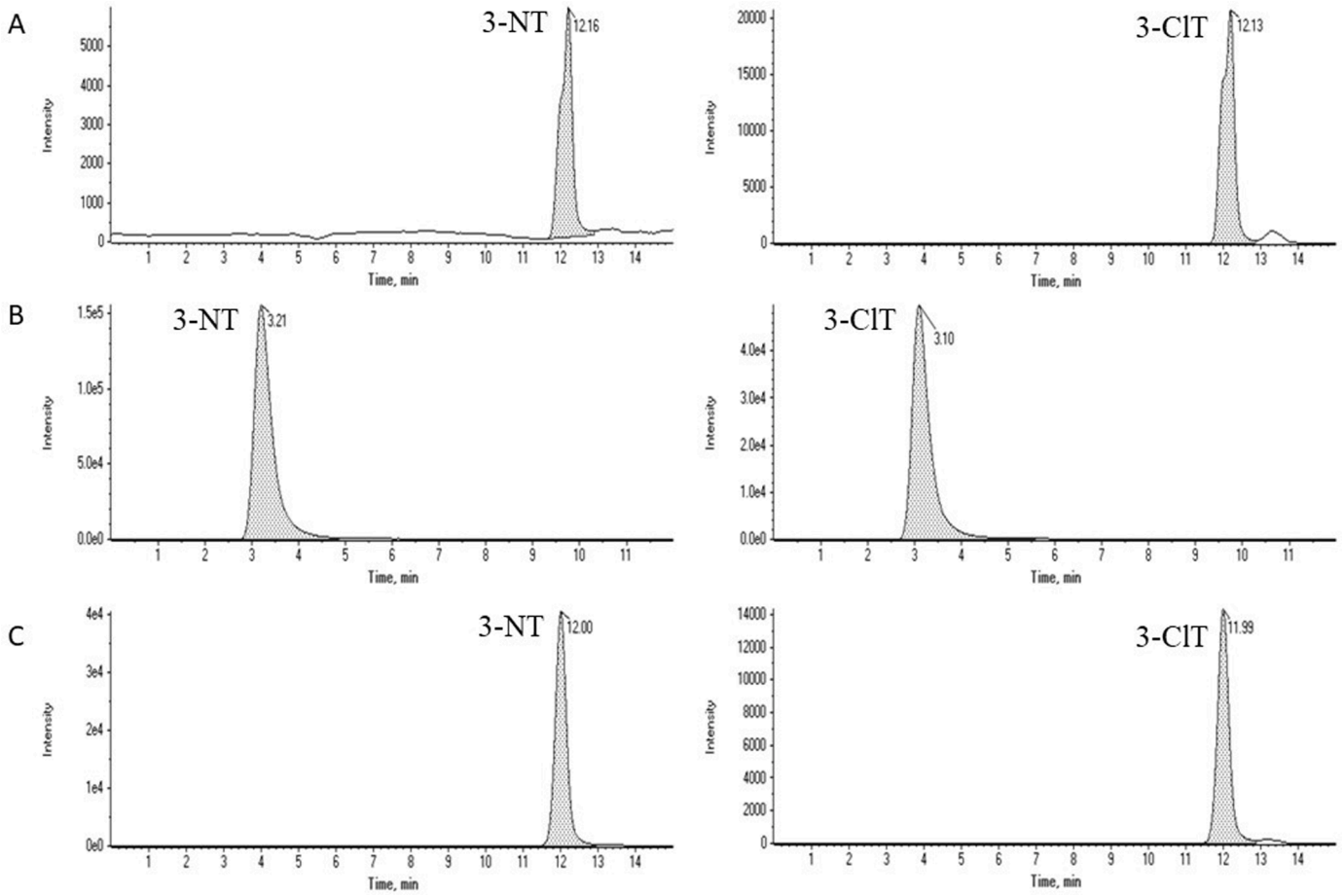

Figure 1. Multiple reaction monitoring (MRM) chromatograms of 3-nitrotyrosine (3-NT) and 3-chlorotyrosine (3-ClT) on a surrogate matrix for quantification in (A) plasma (B) urine and (C) liver tissue.

LOQ was 1.02 ng/mL for plasma, 0.24 mg/L for urine and 0.05 mg/kg for liver tissue. The results of precision, accuracy and recovery are shown in Table 2. Regarding the dilution study, the factor was 1/10 for plasma and liver tissue, and 1/5 for urine. 3-NT was stable in the different matrices after short-term storage for 30 days at -80 °C, after long-term storage for six months at −80 °C, and after three freeze and thaw cycles. The precision for the validated method, dilution study and stability was < 15% and the accuracy was in the range of 85–115 %. The results obtained meet the acceptance criteria established by the reference guides (Houghton et al., 2009; Bioanalytical Method Validation, 2018).

Table 2
Precision, accuracy and recovery for the different matrices.

		Plasma	*Urine*	*Liver tissue*
Accuracy (%)	QC LOQ	110.6	102.0	113.5
	QC low	102.1	118.1	111.5
	QC medium	106.7	93.6	85.2
	QC high	101.3	95.0	94.9
Precision (CV %)	QC LOQ	10.8	1.2	3.4
	QC low	4.4	6.2	8.1
	QC medium	3.9	3.0	2.7
	QC high	4.9	2.5	11.3
Recovery (%)	QC low	106.9	94.7	112.2
	QC medium	93.0	106.9	100.3
	QC high	89.5	101.1	113.9

QC: quality control. LOQ: limit of quantitation. CV: coefficient of variation.

3.2. Milk yield, THI and BCS

The THI for the cows of the WG and SG during the period when the samples were obtained was 55.27 ± 6.48 and 69.94 ± 6.43, respectively. The milk production at 120 d in milk was higher for the WG (4117.72 ± 105.99 L) than for the SG (3583.16 ± 105.993 L) ($P < 0.05$). The BCS decreased throughout the period evaluated ($P < 0.05$), but without differences between groups or interaction effect (Table 3).

3.3. Plasma parameters and liver TAG content

NEFA concentration showed no differences due to the season, time or interaction effect ($P > 0.05$). BHBA concentration was higher on day 7 and 21 postpartum and in the cows of the SG compared to those of the WG ($P < 0.05$). Moreover, BHBA concentration showed a season x time interaction effect, being higher on day 21 postpartum in the cows of the SG. Glucose concentration was lower in the SG than in the WG ($P < 0.05$), without differences due to time or interaction effect ($P > 0.05$). The liver TAG content increased during the postpartum, with a peak on day 7 ($P < 0.05$), without differences between groups. However, the liver TAG content showed a season x time interaction effect, being lower during the prepartum but higher during the postpartum days in cows of the SG ($P < 0.05$).

Regarding liver function biomarkers, bilirubin concentration was higher during the postpartum ($P < 0.05$), and lower in cows of the SG ($P < 0.05$), with no interaction among values ($P < 0.05$). Cholesterol concentration was higher during the postpartum ($P < 0.05$) without differences between groups ($P > 0.05$). Also, a season x time interaction effect was observed, where cholesterol concentration was higher on day

Table 3
Body condition score (BCS), concentration of non-esterified fatty acids (NEFAs), beta-hydroxybutyric acid (BHBA), glucose, albumin, bilirubin, and cholesterol, and aspartate transaminase (AST) and gamma-glutamyl transpeptidase (GGT) activities in plasma and triacylglycerol (TAG) content (mg/g wet tissue) in liver tissue of dairy cattle.

		Prepartum days	*Postpartum days*				
		-21	**7**	**21**	**S**	**T**	**S x T**
BCS	WG	3.36 ± 0.06	2.90 ± 0.08	2.72 ±0 .06	0.54	< 0.01	0.04
	SG	3.47 ± 0.06	3.01 ± 0.05	2.65 ± 0.06			
NEFAs	WG	0.70 ± 0.11	0.73 ± 0.10	0.61 ± 0.09	0.89	0.23	0.11
(mmol/L)	SG	0.50 ± 0.08	0.80 ± 0.13	0.74 ± 0.09			
BHBA	WG	0.29 ± 0.03^{b,A}	1.33 ± 0.41^{a,A}	0.68 ± 0.07 ^{a,B}	< 0.01	< 0.01	< 0.01
(mmol/L)	SG	0.29 ± 0.03^{b,A}	1.54 ± 0.21 ^{a,A}	1.87 ± 0.25 ^{a,A}			
Glucose	WG	52.55 ± 3.60	54.89 ± 5.07	48.72 ± 3.81	< 0.01	0.26	0.86
(mg/dL)	SG	36.43 ± 3.22	34.40 ± 4.21	30.40 ± 2.52			
Albumin	WG	3.38 ± 0.18	3.37 ± 0.17	3.45 ± 0.11	0.41	0.95	0.40
(g/dL)	SG	3.43 ± 0.04	3.39 ± 0.09	3.51 ± 0.13			
Bilirubin	WG	0.72 ± 0.07	0.99 ± 0.07	0.75 ± 0.07	<0.01	<0.01	0.24
(mg/dL)	SG	0.47 ± 0.06	0.79 ± 0.09	0.74 ± 0.10			
Cholesterol	WG	81.94 ± 3.42b^{,A}	84.41 ± 4.26b^{,B}	133.53 ± 6.50^{a,A}	0.12	<0.01	<0.01
(mg/dL)	SG	93.37 ± 5.84^{b,A}	102.00 ± 6.02^{b,A}	130.80 ± 7.70^{a,A}			
AST	WG	64.55 ± 6.61^{b,A}	92.48 ± 6.39^{a,A}	72.39 ± 3.73^{b,B}	0.06	<0.01	<0.01
(IU/L)	SG	69.90 ± 5.49^{c,A}	97.33 ± 12.21^{b,A}	116.98 ± 12.91^{a,A}			
GGT	WG	17.85 ± 1.80	26.69 ± 6.37	26.46 ± 7.05	0.41	0.01	0.10
(IU/L)	SG	22.96 ± 1.25	25.86 ± 2.60	33.64 ± 2.72			
Liver TAG	WG	11.50 ± 1.74^{a,A}	24.66 ± 3.36^{c,B}	17.38 ± 2.69^{b,B}	0.14	< 0.01	< 0.01
(mg/g WT)	SG	5.85 ± 0.43^{a,B}	45.85 ± 8.22^{c,A}	35.37 ± 4.61^{b,B}			

Cows were sampled in winter (WG; n = 16) and spring (SG; n = 16) at 21 days prepartum, and at 7 and 21 days postpartum. Values are expressed as mean ± SEM. The statistical effects of Season (S), Time (T) and S x T are indicated. $^{a-c}$S × T ($P \leq 0.05$) within a given S at different time $^{A-B}$S × T ($P \leq 0.05$) between seasons at a given week.

7 postpartum in cows of the SG. Albumin concentration showed no differences between groups due to time or interaction effect ($P > 0.05$).

AST activity was higher in the postpartum ($P < 0.05$), without differences between groups ($P > 0.05$). In addition, AST activity showed a season x time interaction effect, where the enzyme activity first increased on day 7 and then returned to the prepartum level on day 21 in cows of the WG. In cows of the SG, AST activity was higher on day 21 postpartum than on day 7 postpartum ($P < 0.05$). GGT activity was higher on day 21 postpartum than on day 21 prepartum ($P < 0.05$), without differences between groups or interaction effect ($P > 0.05$) (Table 3).

3.4. 3-NT concentration in plasma, urine and liver tissue

Plasma 3-NT concentration was lower during the postpartum period, being higher in cows of the SG ($P < 0.05$), but without interaction effect ($P > 0.05$). In urine, 3-NT concentration was higher on day 21 postpartum in cows of the SG ($P < 0.05$), and a season x time interaction effect was observed. In cows of the WG, 3-NT concentration showed no differences due to time ($P > 0.05$), whereas in cows of the SG, 3-NT concentration was higher on day 21 postpartum ($P < 0.05$). Finally, in liver tissue, 3-NT concentration was lower during the postpartum period, being higher in cows of the SG ($P < 0.05$), but without interaction effect ($P > 0.05$) (Figure 2).

Regarding the agreement between 3-NT concentration in the different matrices, a high correlation was observed between 3-NT concentration in urine and liver ($r = 0.74$; $P < 0.01$) and a moderate correlation between 3-NT concentration in plasma and urine ($r = 0.61$; $P < 0.01$) and between that in plasma and liver ($r = 0.55$; $P < 0.01$) (Table 4).

4. Discussion

The transition period, which is the most critical in the productive cycle of dairy cows, causes great metabolic and endocrine changes, particularly a negative energy balance and hypocalcemia (Barletta et al., 2017; Neves et al., 2017). In addition, during this period, cows may suffer OS, a phenomenon that can increase the susceptibility of animals to develop several postpartum diseases (Sordillo and Aitken, 2009). Hence, several studies have evaluated the antioxidant capacity in cows (Celi, 2011; Sayiner et al., 2021) and parameters which could quantify OS. In this context, 3-NT has been described as a marker of inflammation, OS and nitrosative stress (Bandookwala et al., 2020). To quantify this biomarker, it is important to know that it exists in two forms: as a free amino acid and as a constituent amino acid within a protein structure. However, the quantification of protein-bound 3-NT can lead to the generation of artifacts in biological samples because the treatment of the samples involves a hydrolysis method. Some authors have reported that, during treatment, the sample is subjected to various factors such as chemical or enzymatic modification, exposure to reduced pH and/or high temperatures, resulting in the formation of artifacts. On the other hand, free 3-NT has been detected by different analytical techniques and most extensive research has been performed in humans due to its clinical importance (Yi et al., 2000; Radabaugh et al., 2008; Li et al., 2015; Fleszar et al., 2020).

In dairy cows, the only study so far reported regarding the quantification of 3-NT in plasma was through ELISA (Cigliano et al., 2014), and, to our knowledge, no study has quantified free 3-NT in any matrix with LC-MS/MS. Therefore, in this study, we developed and validated a LC-MS/MS method to quantify free 3-NT in bovine plasma, urine and liver tissue by using a surrogate matrix calibration curve. For each matrix, the method was linear for the proposed analytical ranges, being

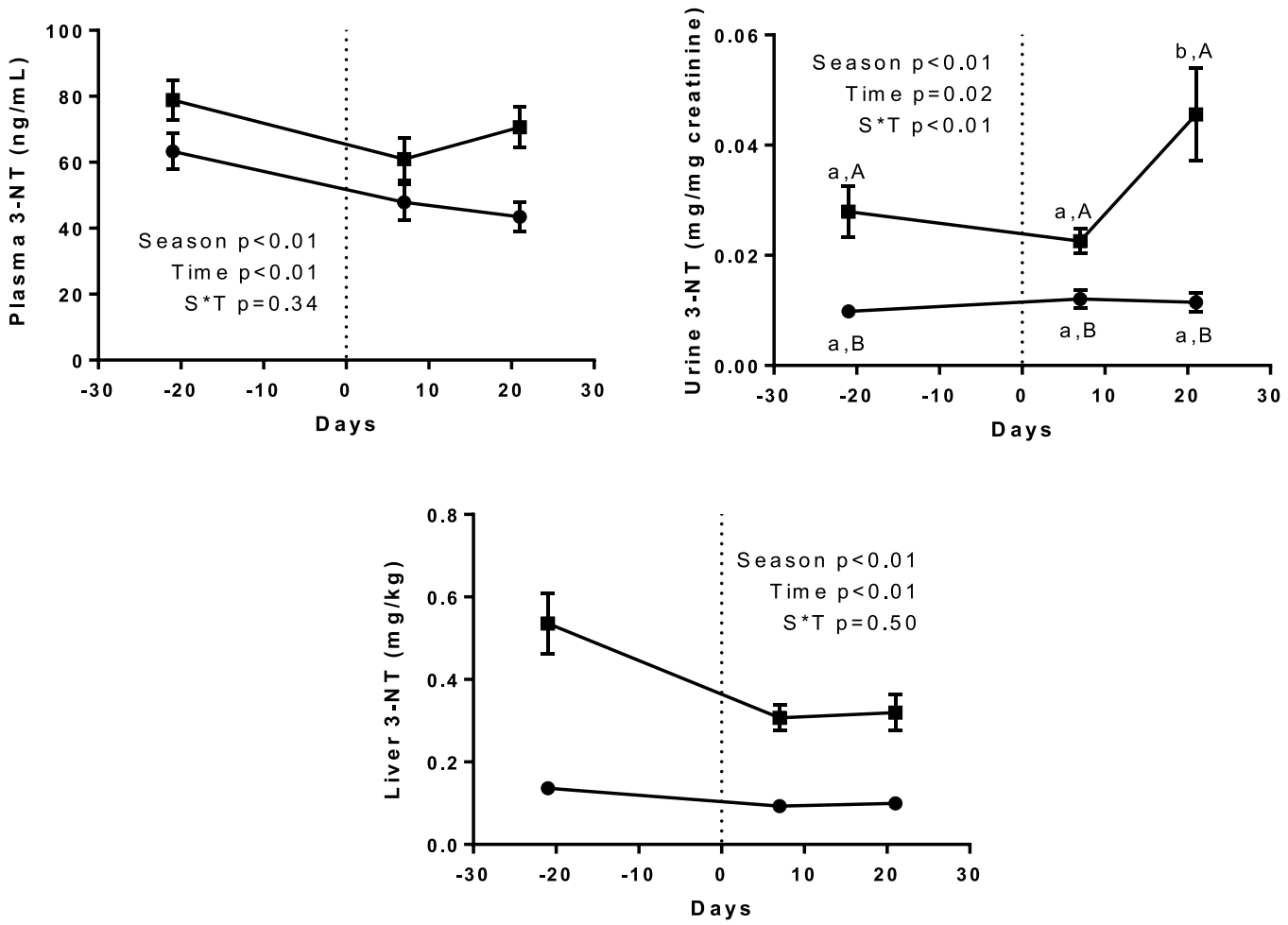

Figure 2. Concentration of 3-NT in plasma, urine and liver samples from dairy cattle. Cows were sampled in winter (WG; n = 16; black circles) and spring (SG; n = 16; black squares) at 21 days prepartum, and at 7 and 21 days postpartum. Values are expressed as mean ± SEM. The statistical effects of season (S), time (T) and S x T interaction are indicated. a–cS × T ($P \leq 0.05$) within a given S at a different time; A-BS × T ($P \leq 0.05$) between seasons at a given week. 3-NT: 3-nitrotyrosine.

Table 4
Statistical results for the relation between 3-NT concentration in plasma, urine and liver tissue.

	Plasma	*Urine*	*Liver tissue*
Plasma		r = 0.612 (P < 0.01)	r = 0.547 (P < 0.01)
Urine	r = 0.612 (P < 0.01)		r = 0.740 (P < 0.01)
Liver tissue	r = 0.547 (P < 0.01)	r = 0.740 (P < 0.01)	

r: Spearman's correlation coefficient.

specific, accurate and precise, and complying with international regulatory guidelines. This study is the first to describe the development of a selective analytical method to measure the concentrations of free 3-NT by using LC-MS/MS in biological samples from dairy cows.

3-NT was determined in two groups: one that gave birth in winter and another that gave birth in spring. The dairy farm where the samples were taken from is placed in the central region of Argentina, where the THI from October to March is usually above the comfort levels for livestock (Recce et al., 2021). Although some studies have described that, in order to cause heat stress, the THI values have to be higher than 72, other studies have indicated that a THI value of 68 can negatively affect high-producing dairy cows (Huber et al., 2020). According to the above, it is important to highlight that, in our study, cows of the SG had a THI value of 69. On the other hand, it is known that heat stress is related to decreased dry matter intake (DMI) and milk yield (West, 2003). In our study, although the animals from both groups did not receive the same diet during the postpartum, the energy and protein concentration as well as the mineral supplementation were not different between groups. In addition, cows of the SG had lower milk yield than cows of the WG. Also, cows of the SG showed higher BHBA concentration and liver TAG content and lower glucose concentration than the cows of the WG, which could indicate a greater negative energy balance in the animals of the SG, in concordance with previous studies (Turk et al., 2015; Zachut et al., 2017). Although differences in NEFA concentration have not been observed between groups, previous studies from our group have described that a lower plasma BHBA concentration and liver TAG content in cows could indicate a greater adaptation to the postpartum lipid mobilization (Angeli et al., 2019a). In this sense, the differences observed may also be due to metabolic changes which could affect the postpartum adaptation, particularly the hepatic adaptation and not only to a lower DMI. Moreover, previous studies have shown metabolic alterations in control animals subjected to heat stress with the same DMI (Lamp et al., 2015; Koch et al., 2016). Regarding this, the increase in the hepatic TAG content in cows of the SG, which could explain a lower hepatic adaptation, was observed prior to the systemic increase in BHBA recorded in cows of the SG group with respect to cows of the WG. In addition, the lower glucose concentration observed in cows of the SG was recorded throughout the period evaluated, including 21 days before calving, when the cows were not in negative energy balance. In this sense, Zachut et al. (2017) described that the biological mechanism underlying the action of heat stress includes an altered endocrine state, reduced rumination and nutrient absorption, and higher maintenance

requirements.

To gain further insights into the hepatic adaptation and health of dairy cows in the transition period, we also evaluated some liver functionality parameters. AST activity was lower in cows of the SG on day 21 postpartum, and this was related to the higher liver TAG content, which could suggest alterations in the integrity of hepatocytes. However, the concentrations of cholesterol and bilirubin were higher and lower, respectively, in cows of the SG, without a relation with AST activity. Other studies have demonstrated no association between tissue damage parameters, such as enzymes and others related to liver function in postpartum cows (Bionaz et al., 2007; Bertoni et al., 2008; Angeli et al., 2019b). Also, it is important to note that the animals of our study belong to different herds. In this regard, Bertoni and Trevisi (2013) considered albumin, bilirubin and cholesterol to compare different herds but developed a functionality index with samples obtained from the same animal at 3 and 28 days postpartum.

In both ruminants and non-ruminants, OS has been related to pro-inflammatory and insulin resistance states (Pouvreau et al., 2018; Angeli et al., 2021). In this context, the evaluation of the biomarker 3-NT in different tissues to analyze the degree of OS could be useful in dairy cows to obtain information to better understand the postpartum adaptation of animals.

In the present study, in the three matrices studied, 3-NT concentration was higher in cows of the SG than in those of the WG. It has been described that an increase in milk yield could increase OS (Abuelo et al., 2015). However, in our study, the cows of the SG had a lower milk yield, in accordance with previous reports related to higher heat stress (West, 2003). The diet could also affect the OS (Abuelo et al., 2015). However, as previously described, the energy and protein concentration, as well as the mineral supplementation received by both groups, were not different in the postpartum diet. Furthermore, although the prepartum diet was not different between groups, the 3-NT concentration in all the matrices was different on day 21 prepartum. For this reason, large part of the differences found in 3-NT concentration could be a consequence of the different temperatures to which the animals were exposed. Previous studies have shown alterations in other plasma OS biomarkers during summer, suggesting that heat stress enhances several stress-related pathways, manifested by higher OS related to an increased inflammatory state (Turk et al., 2015; Zachut et al., 2017; Guo et al., 2021). In our study, 3-NT concentration in urine showed a pattern slightly different from that observed in plasma, showing an increase on day 21 postpartum in the cows of the SG. In this sense, a moderate correlation was observed between plasma and urine 3-NT concentration. Urine samples could be interesting because they are easier to obtain and because, in dairy farms, they are routinely collected during the prepartum period for pH evaluation. Moreover, the changes observed in urine could indicate greater sensitivity to detect changes in OS. However, prior to elimination in the urine, part of 3-NT is metabolized to 3-nitro-4-hydroxyphenylacetic acid, which can lead to incorrect conclusions when urinary concentrations are extrapolated to the systemic pattern (Chao et al., 2015). Also, other human studies related to arthritis and joint injury have described that 3-NT concentration in urine was not well correlated with these diseases (Misko et al., 2013).

Regarding liver 3-NT, in our study, we observed a higher concentration in cows of the SG than in cows of the WG, in concordance with the 3-NT concentration recorded in plasma. However, a moderate correlation was observed between plasma and urine 3-NT concentration. During the transition period, the liver is relevant because it deals with the higher influx of NEFAs (Angeli et al., 2019b). On the other hand, in ruminants, the main source of glucose comes mainly from gluconeogenesis, so the liver must adapt as soon as possible to contribute to the increased demand for glucose and thus achieve high milk production (Drackley et al., 2001). Therefore, in this metabolic context, the increase in OS could compromise the liver health. Furthermore, the high concentration of 3-NT in the liver from cows of the SG was even higher on 21 day prepartum, when no significant hepatic metabolism is expected, so this increase in SG compared to WG could not be associated with an increased metabolism. Probably, the increased OS in cows could collaborate with the increases recorded in AST activity and TAG content during the postpartum period. In this sense, Strickland et al. (2019) described that cows with high hepatic copper concentrations had higher liver 3-NT concentrations but no evidence of active liver disease.

5. Conclusion

In recent years, some authors have focused on the OS of dairy cows, but probably with limitations to quantify and evaluate it in tissues and fluid samples from animals. This study describes the development and validation of an LC-MS/MS method for the quantification of an important OS biomarker in fluids and tissue from two groups of animals under different environmental conditions, showing that it could be a very useful parameter to evaluate the comfort of dairy cows, especially during the transition period. Nevertheless, more studies are needed to determine cut-off points, especially taking into account the different metabolic states of ruminants and in particular of dairy cows.

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CRediT authorship contribution statement

D. Barcarolo: Conceptualization, Methodology, Software, Data curation, Writing – original draft. **E. Angeli:** Visualization, Investigation, Writing – original draft. **L.E. Ribas:** Conceptualization, Methodology, Software, Data curation, Writing – original draft. **S.M. Addona:** Conceptualization, Methodology, Software, Data curation, Writing – original draft. **H.H. Ortega:** Supervision, Writing – review & editing. **G. J. Hein:** Supervision, Writing – review & editing.

Conflict of interest statement

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial for this work that could have influenced its outcome. I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of them. I confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing I confirm that we have followed the regulations of our institutions concerning intellectual property. I understand that as Corresponding Author I am the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). I am responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. I confirm that I have provided a current, correct email address (in the cover letter also): ghein@santafe-conicet.gov.ar

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